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Campylobacter N-glycan presenting *Salmonella Typhimurium*: a new vaccine for broiler chickens?

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List of abbreviations

A	ampicillin
AB	Applied Biosystems
ATP	adenosine-5'-triphosphate
Bac	bacillosamine
B-cells	bursa of fabricius cells
BPW	buffered peptone water
CC	clonal complex
C. coli	*Campylobacter coli*
CD	cluster of differentiation
cfu	colony forming units
CH	Switzerland
C. jejuni	*Campylobacter jejuni*
COL SB	columbia agar with 7 % sheep blood
CRL	community reference laboratory
Δ	deletion mutant
ECA	enterobacterial common antigen
ECA_{LPS}	enterobacterial common antigen linked to LPS
ECL	enhanced chemiluminescense
E. coli	*Escherichia coli*
EFSA	european food safety authority
ELISA	enzyme linked immunosorbent assay
FAM	6-carboxyfluorescein
FIT	federal institute of technology
FVO	federal veterinary office

GalNAc	*N*-Acetylgalactosamine
GlcNAc	*N*-Acetylglucosamine
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
Glc	glucose
GBS	Guillain-Barré syndrome
3D5-glyc	glycosylated scFv 3D5
H	H-antigen
HCl	hydrochloric acid
HEX	5'-hexachlorofluorescein
his	histidine
HR	horseradish
IB	immunoblot
Ig	immunoglobulin
IgA	immunoglobulin A
IgG	immunoglobulin G
IgM	immunoglobulin M
IgY	immunoglobulin Y
IL	interleukin
IPC	internal positive control
ISO	international organization for standardization
IVB	institute of veterinary bacteriology
kb	kilobase
kDa	kilodalton
LB agar	lysogeny agar syn. Luria-Bertani
LB broth	lysogeny broth syn. Luria-Bertani

LB-ST agar	LB agar containing streptomycin and tetracycline
LB-STA agar	LB agar containing streptomycin, tetracycline and ampicillin
LB-STA broth	LB broth containing streptomycin, tetracycline and ampicillin
LOS	lipooligosaccharide
LPS	lipopolysaccharide
LT	heat-labile enterotoxin
M-cells	microfold cells
MSRV plates	modified semi-solid Rappaport Vassiliadis agar plates
nd	not done
NENT	national reference center for enteropathogenic bacteria and *Listeria monocytogenes*
NL	The Netherlands
n.s.	not significant
OD	optical density
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
pgl operon	protein glycosylation operon
PglB	PglB oligosaccharidetransferase in *Campylobacter*
pgl+ *E. coli*	glycosylation competent *E. coli*
PglH	PglH transferase in *Campylobacter*
PglK	ATP binding cassette transporter PglK in *Campylobacter*
*pgl*mut	mutant protein glycosylation operon from *C. jejuni*
p.i.	*post infectionem*
PVDF	polyvinylidenfluorid
R phenotype	rough phenotype of *Salmonella*

S phenotype	smooth phenotype of *Salmonella*
S	streptomycin
S. Typhi	*Salmonella enterica* subsp. *enterica* serovar Typhi
S. Typhimurium	*Salmonella enterica* subsp. *enterica* serovar Typhimurium
scFv	single-chain variable fragment
sIgA	secretory IgA
SKI40	vaccine strain in this study
SKI41	backbone strain in this study
SNP	single-nucleotide polymorphism
sp.	species (singular)
spp.	species (plural)
ST	sequence type
T	tetracycline
TAMRA	6-carboxytetramethylrhodamine
T-cells	thymus cells
TGF-β	transforming growth factor β
TRIS-HCl	tris(hydroxymethyl)aminomethane hydrochloric acid buffer
TTBS	TRIS buffered saline + Tween20
Tween20	nonionic polyoxyethylene surfactant
UDP	undecaprenyl phosphate
VIC	compound name not released by Applied Biosystems
WaaL	O-antigen ligase
WbaP	WbaP transferase in *Salmonella*
WecA	WecA transferase in *Salmonella*
WHO	world health organisation

Wxz	Wxz “O-antigen flippase” in *Salmonella*
Wzy	Wzy polymerase in *Salmonella*
ZOBA	national reference centre for zoonoses, bacterial animal diseases and antimicrobial resistance

1. Summary

1.1. Summary

Campylobacteriosis is an emerging foodborne enteric disease in humans worldwide. Poultry meat is an important source of infection. One strategy in the combat against *Campylobacter* at production level is the vaccination of broiler chickens. In this study, a *Salmonella enterica* subsp. *enterica* serovar Typhimurium (*S.* Typhimurium) strain was modified and used as a vector to express the *N*-glycan from *Campylobacter* on its surface. The immunogenicity of this live vaccine was tested *per os* in 70 commercial broilers. The vaccine was able to colonize broilers without causing disease. No detectable humoral immune response and no reduction of colonization of *C. jejuni* in the caeca was observed due to vaccination, however the chickens produced antibodies against the *Campylobacter N*-glycan upon infection with the *Campylobacter jejuni* challenge strain.

Keywords: *Campylobacter N*-glycan, live-attenuated *Salmonella* Typhimurium vaccine, humoral immune response, broiler chickens

1.2. Zusammenfassung

Die Campylobacteriose des Menschen ist eine weltweit vorkommende, lebensmittel-assozierte gastrointestinale Erkrankung. Der Einsatz eines geeigneten Impfstoffes ist eine mögliche Strategie, um die *Campylobacter*-Besiedlung des Mastgeflügels zu reduzieren. In dieser Studie wurde als Vektor *Salmonella enterica* subsp. *enterica* serovar Typhimurium (*S.* Typhimurium) verwendet, welche das *Campylobacter N*-Glykan als spezifisches Antigen auf der Oberfläche präsentiert. Dieser Lebendimpfstoff wurde kommerziellen Masttieren per oral verabreicht und die Immunogenität und die Verträglichkeit getestet. Der Impfvektor konnte die Blinddärme der Masttiere kolonisieren, ohne eine Erkrankung hervorzurufen. Die Impfung induzierte indes keine humorale Immunantwort gegen das präsentierte *Campylobacter* Antigen und führte auch zu keiner Reduktion der *C. jejuni* Kolonisation in den Blinddärmen der Versuchsbroiler. Die Infektion mit *C. jejuni* in den Challenge Experimenten führte jedoch wie bei einer natürlichen *Campylobacter* Infektion zu einer erworbenen humoralen Immunantwort gegen das *Campylobacter N*-Glykan.

Stichworte: attenuierter *Salmonella* Typhimurium Lebendimpfstoff, Mastgeflügel, *Campylobacter N*-Glykan, humorale Immunantwort

2. Introduction

Campylobacter jejuni (*C. jejuni*) and the closely related *Campylobacter coli* (*C. coli*) are thermophilic gram-negative bacteria. They belong to the most important zoonotic agents causing foodborne illness in humans worldwide (Allos 2001, Janssen et al. 2008, Dasti et al. 2010). In Europe, *Campylobacter* is the most reported foodborne pathogen followed by *Salmonella*. In 2009, 45.6 per 100 000 population confirmed campylobacteriosis cases were reported in Europe (EFSA 2011).

Thermophilic *Campylobacter* spp. are ubiquitous in the environment and colonize the alimentary tract of wild and domestic animals as a commensal organism (Newell and Fearnley 2003). Wild birds and chickens are a natural *C. jejuni* / *C. coli* reservoir (Newell and Fearnley 2003, Janssen et al. 2008, Dasti et al. 2010). The bacteria are primarily found in the mucus covering the epithelia of the caeca and the small intestine (Newell and Fearnley 2003). In general, chickens become colonized the earliest at 10 days of age, and most flocks become infected at the age of two or three weeks. This persistent negative delay of colonization is referred to as lag-phase (Newell and Fearnley 2003, Cawthraw and Newell 2010). Chicken colonization proceeds without clinical symptoms, even when a high infective dose is given (Wyszynska et al. 2004). Nevertheless, Welkos et al. (1984) observed that newly hatched chickens developed enteritis after oral gavage of a *C. jejuni* strain within the first hours. In animal experiments, the infective dose was found to be lower than 40 colony forming units (cfu) of *C. jejuni*, and up to 10^9 cfu per gram caecal contents could be detected in experimentally challenged birds after colonization (Newell and Fearnley 2003, Dasti et al. 2010, Welkos et al. 1984). Bird to bird transmission occurs rapidly. Once the bacterium is introduced into a flock up to 100 percent of the chickens are colonized after a few days due to coprophagy (Brendtson et al. 1996, Newell and Fearnley 2003).

Campylobacter is highly infectious for humans and a very low infective dose of 500 to 800 cfu has been reported (Janssen et al. 2008). Handling of contaminated poultry products and eating of undercooked poultry meat is an important source of sporadic *Campylobacter* infection (Harris et al. 1986, Butzler and Oosterom 1991, EFSA Journal 2010). Sources of massive outbreaks of infection are contaminated drinking water or milk (Butzler and Oosterom 1991, Harris et al. 1986, Janssen et al. 2008, EFSA Journal 2010). Campylobacteriosis is further a frequently reported disease of travellers (Büttner and Jost 2011). Usually, *Campylobacter* infection results in a self-limiting disease presenting clinical symptoms of an acute gastroenteritis characterized by watery or blood-tinged diarrhoea, nausea, abdominal pain and fever (Butzler and Oosterom 1991, Janssen et al. 2008). Nevertheless, a known post-infectious complication due to *Campylobacter* infection is an autoimmune disorder named Guillain-Barré syndrome (GBS) (Hughes et al. 1999).

Because contaminated poultry meat represents a high risk for *Campylobacter* infection (Harris et al. 1986, Butzler and Oosterom 1991, EFSA 2010, Büttner and Jost 2011), industrialized countries attempt to achieve reduction or even elimination of these bacteria in the food chain (Newell and Fearnley 2003, EFSA 2011). Intervention can be made at production level, in the abattoir, and at the consumer level. One strategy at production level is to prevent broiler chickens from being colonized by *Campylobacter* in the first place (Newell et al. 2011, Pasquali et al. 2011, EFSA 2011, Rice et al. 1997). For example, this was achieved with the *Salmonella* eradication in Swiss flocks. In a Danish study (Hald et al. 2007), it was demonstrated that fly screens significantly reduce *Campylobacter* occurrence in broiler houses. However, this measure is not feasible in all husbandry systems. Contamination or cross-contamination of carcasses can be targeted in the abattoir with chemical or physical methods (EFSA 2011). The cross-contamination in the consumer's kitchen is also an important risk factor. Contamination occurs by way of direct contact of raw meat or meat

juice with products that are eaten uncooked or by indirect path, e.g. via work surface, hands or cooking utensils (Jacobs-Reitsma et al. 2008, EFSA 2010).

One of the most promising and effective strategies to avoid *Campylobacter* colonization in chickens is to vaccinate against these commensal bacteria. De Zoete et al. (2007) mentioned that vaccination of chickens against *Campylobacter* bears the challenge of identifying a cross-protective antigen, which is able to induce an early and potent immune response.

Many efforts have already been taken in production of vaccines, however with limited success (as summarized in De Zoete et al. 2007). In one study, mice were orally vaccinated with heat-killed *Campylobacter* in combination with heat-labile enterotoxin (LT) from *Escherichia coli.* These mice developed a protection against *Campylobacter* infection and elicited a secretory IgA (sIgA) immune response against particular *Campylobacter* antigens (Rollwagen et al. 1993, Baqar et al. 1995). A similar vaccine experiment was done with broiler chicks. Birds received formalin inactivated *C. jejuni* with or without *E. coli* LT *per os*. A reduction of the caecal load of *C. jejuni* compared with the non-vaccinated control was reported and further development of an orally applicable vaccine was recommended (Rice et al. 1997).

Attenuated *Salmonella* spp. mutants are recognized as outstanding organisms to generate a live recombinant vaccine, which can achieve a mucosal immunization in the vaccinated livestock (Kotton and Hohmann 2004). An avirulent *Salmonella* carrying the *C. jejuni* 72Dz/92 *cja*A gene was used for a vaccine study, demonstrating a reduction of *C. jejuni* in the caeca of the birds in protection experiments (Wyszynska et al. 2004). Layton et al. (2011) used an attenuated *Salmonella* vector expressing three peptide epitopes from *Campylobacter* proteins on the surface, and demonstrated a reduction of the *Campylobacter* load in the ileum.

Not only proteins may be used as antigens. In the search for new cross-protective antigens for vaccine development, oligosaccharide molecules offer a new, promising approach.

O- or *N*-linked protein glycosylation in eukaryotes is a universal posttranslational modification and influences protein functions, e.g. stabilisation of the native state, immune response modulation and solubility enhancement (Helenius and Aebi 2004, Szymanski and Wren 2005, Nothaft and Szymanski 2010). The newly discovered prokaryotic *N*-linked protein glycosylation pathway was first described in *C. jejuni* and is now well characterized (Linton et al. 2005, Wacker et al. 2002, Young et al. 2002). The genes located on the 16 kb long *pgl* loci, are responsible for the posttranslational modifications of over 65 periplasmatic and surface proteins (Wyszynska et al. 2008, Szymanski and Wren 2005, Nothaft and Szymanski 2010). *N*-linked glycosylated proteins in *Campylobacter* are known to be highly immunogenic in humans (Szymanski et al. 1999, Hermans et al. 2011). Wacker et al. (2002) demonstrated that the *pgl* operon from *C. jejuni* could be transferred into *E. coli*, which is subsequently able to produce *N*-linked glycoproteins. These *E. coli* strains are designated "glycosylation competent" (*pgl*+) *E. coli* and are particularly useful in glycoengineering. Glycoengeneering aims at producing glycosylated proteins in high purity and of considerable yield, as shown by Lizak et al. 2011, who used *pgl*+ *E. coli* to glycosylate and produce the murine single-chain variable fragment (scFv) from anti-His tag antibody 3D5 (characterised and engineered by Kaufmann et al. 2002).

Little is known about the biological significance of protein glycosylation in bacteria. However, a chicken colonization study with a *Campylobacter pgl*H (transferase) mutant showed reduced ability of the mutant to colonize chickens (Karlyshev et al. 2004). Mutants in the *N*-linked *pgl* system further lead to changes in protein antigenicity, reduced cell invasion and reduced capacity to colonize the intestinal tract of mice and chickens (Szymanski et al. 2002, Szymanski et al. 2003, Larsen et al. 2004)

The building blocks of the *C. jejuni* glycan are *N*-acetylgalactosamine (GalNAc), glucose (Glc) and bacillosamine (Bac; 2,4-diacetamido-2,4,6-trideoxy-glucopyranose) (Szymanski et

al. 1999, Young et al. 2002, Szymanski and Wren 2005) The assembled *C. jejuni* heptasaccharide consists of Bac-GalNAc$_5$-Gluc (Table 2), with a bacillosamine at its reducing end (Young et al. 2002). This entity was chosen as candidate antigen for a vaccine in broilers because it is highly conserved in *Campylobacter* (Szymanski and Wren 2005) and is therefore thought to elicit a good cross-protectivity. Vaccination in broiler chicken should be broad concerning *Campylobacter* species (De Zoete et al. 2007), because vaccinating against one species may open a niche for another. As immunogenicity of this specific *Campylobacter N*-glycan was proven in sera from humans and mice, the use of this antigen is very promising (Szymanski et al. 1999, Ilg 2009).

As a prerequisite to our study, a newly engineered *Salmonella enterica* subsp. *enterica* serovar Typhimurium (*S.* Typhimurium*)* vector strain was constructed. To understand how this *Salmonella* was tweaked to highly express *Campylobacter*-heptasaccharide, we first have to briefly get acquainted to the biosynthesis of glycolipids in *Salmonella* and secondly to the *Campylobacter N*-glycan pathway.

Like all Gram-negative bacteria, a wild type *S.* Typhimurium has an inner and an outer membrane. Within the family *Enterobacteriaceae*, the outer membrane carries the lipopolysaccharides (LPS) and the enterobacterial common antigen (ECA). LPS consists of a lipid A core, non-repetitive oligosaccharide and a repetitive polysaccharide, the latter being species-specific and commonly known as O-antigen (Raetz und Whitfield 2002, Murray et al. 2003). The O-antigen together with the H-antigen of the flagellum forms the basis of *Salmonella* serotyping, according to the White-Kauffmann-Le Minor scheme (Grimont and Weill 2007). *Salmonella* with LPS show a smooth (S) phenotype when cultured on agar plates, while LPS-lacking *Salmonella* show a so-called rough (R) phenotype, that is recognized by flatter, rougher colonies with frayed margins. Not all steps in the assembly of LPS in *Salmonella* and its shuttling to the surface of the outer membrane are understood. The

current hypothesis is that the lipid A core is synthesized in the cytoplasm, shuttled to the outer membrane, and anchored in the outer membrane. The variable part of the LPS is assembled in the cytoplasm and initially processed by the WbaP transferase, transported through the inner membrane by a Wxz-dependent mechanism and polymerized by Wzy. Wxz is thus colloquially known as "O-antigen flippase" (Liu et al. 1996). ECA is an outer membrane glycolipid produced by all *Enterobacteriaceae* (Makela and Mayer 1976). ECA_{LPS} uses a pathway initiated by the transferase WecA to be attached to a lipid A core in the outer membrane.

For *N*-linkage of different proteins in *Campylobacter*, the heptasaccharide is assembled on a lipid carrier in the inner cell membrane (undecaprenyl pyrophosphate), shuttled through the inner membrane by the ATP binding cassette (ABC) transporter PglK and attached to its acceptor proteins by the PglB oligosaccharide transferase (Feldman et al. 2005, Szymanski et al. 2003).

The *S.* Typhimurium O-antigen ligase WaaL, which transfers the polymerised O-antigen to the lipid A core, shows relaxed saccharide substrate specificity (Falt et al. 1996, Wang et al. 1999, Xu de et al. 2007). Thus it is possible to engineer a *Salmonella* linking a *Campylobacter N*-glycan to the lipid A core on its surface. For this, an O-antigen negative derivate of the *S.* Typhimurium SL1344 (a human isolate by Hoiseth and Stocker 1981) termed SL1344ΔwbaP (SKI12) (Ilg et al. 2009) could be transformed with a plasmid containing a modified *C. jejuni pgl* cluster (termed *pgl*3mut), where N-acetylglucosamine (GlucNAc) gets incorporated into the heptasaccharide instead of bacillosamine. Additionally, *pgl*3mut contains an inactive oligosaccharyltransferase PglB. This construct results in an O-antigen negative *S.* Typhimurium displaying the *C. jejuni* heptasccharide, with a GlucNAc instead of a bacillosamine at the reducing end. This GlucNAc at the reducing end is introduced due to enhanced acceptor activity of WecA, as ECA assembly also occurs on

undecaprenylpyrophosphate, the carrier of the N-glycan. This leads to a high amount of the antigen displayed on the *Salmonella* surface (Ilg 2009).

To test the immunogenicity of the modified *C. jejuni* heptasaccharide presented by the *S.* Typhimurium SKI12, three 6-9 weeks old female C57BL/6 mice were injected intravenously with the heat-inactivated SKI12. One of three mice showed specific anti *Campylobacter N*-glycan IgG in immunoblot analysis (Ilg 2009).

In order to be used in this study with chickens, minor changes were performed on the *S.* Typhimurium strain SKI12, precise integration of the *pgl*-operon into the genome was performed. The resulting strain was named SKI40. A corresponding strain, harbouring the vector control, lacking the *pgl* insert, was constructed and named SKI41. Note that the phenotype of SKI40 and SKI41 is a R-form, because it is devoid of the O-antigen.

In this study we tested whether *S.* Typhimurium SKI40, presenting the *Campylobacter*-heptasaccharide on its surface, is a suitable candidate for a vaccine against *Campylobacter* in broiler chickens. We wanted to address the following points: (i) evaluate the attenuation of the *Salmonella* vector in an avian species, (ii) test the stability of the *pgl*3mut *in vivo*, (iii) have insight into the humoral immune response against entire and truncated forms of the *Campylobacter*-heptasaccharide and the *Salmonella* backbone in vaccinated chickens and (iv) measure whether the vaccine leads to reduction in *Campylobacter* colonization of the caeca.

3. Materials and Methods

3.1. Bacteriology

3.1.1. Bacterial strains

An overview of bacterial strains used in this study is given in Table 1.

3.1.2. *Salmonella* detection by culture

Qualitative detection in animal faeces, organs and environmental samples was carried out according to EU 2002. Briefly, samples were incubated in buffered peptone water ("Buffered Peptone Water (ISO)" (BPW), CM1049; Oxoid, Wesel, Germany) for 24 h at 37 °C, subcultivated onto modified semi-solid Rappaport Vassiliadis medium ("MSRV Medium Base, modified", 1.09878.0500, "MSRV Selective supplement", 1.09874.0001, Merck, Darmstadt, Germany) and incubated at 41.5 °C for 18 h. Suspected swarming halos were subcultivated onto xylose lysine deoxycholate agar plates (43563, BioMérieux, Mary L'Etoile, France). *Salmonella* Typhimurium colonies of either SKI40 or SKI41 were recognizable by their rough texture, as these strains are devoid of the O-antigen. Colonies were confirmed with *S.* Typhimurium specific real-time PCR.

Enumeration of the vaccine strains *S.* Typhimurium SKI40 and SKI41 in caecal contents of experimentally vaccinated chickens was performed by serial dilution in sterile, physiological sodium chloride solution and then plated in duplicates on Luria-Bertani (LB) agar (244520, Difco Laboratories, Detroit, MI, U.S.A) plates containing streptomycin (final concentration: 50 µg/ml, A1852, AppliChem, Darmstadt, Germany), tetracycline (final concentration: 10 µg/ml, A2228, AppliChem) and ampicillin (final concentration: 100 µg/ml, A0839, AppliChem). This agar medium will be designated LB-STA agar throughout the text.

Stability testing of the pBR*pgl*3mut plasmid of SKI40, and the pBR322 plasmid of SKI41, which both carry an ampicillin resistance (Table 1), was performed using caecal content taken after necropsy. Serial 10-fold dilutions of faecal samples were cultured on LB-STA and on

LB agar containing only streptomycin (final concentration: 50 μg/ml) and tetracycline (final concentration: 10 μg/ml), designated LB-ST agar.

3.1.3. *Campylobacter* detection by culture

Campylobacter detection and enumeration was modified from EU 2006. Briefly, 500 μl sterile sodium chloride suspension containing 50 ng caecal contents was prepared. 50 μl of the serial dilution was plated in duplicates on *Brilliance*^{TM} CampyCount agar plates (PO 5305A, Oxoid) and incubated at 42 °C under microaerobic conditions (5 % O_2, 5 % CO_2, 80 % N_2, and 10 % H_2) for 48 h. Confirmation was done by specific *C. jejuni* real-time PCR.

3.1.4. Production of the vaccine dose

Frozen stock cultures of *Salmonella* Typhimurium strains SKI40 and SKI41 were reconstituted on Columbia agar with 7 % sheep blood (COL SB, PB 5008A, Oxoid, Wesel, Germany) and incubated aerobically at 37 °C for 24 hours, before streaking onto LB-STA agar plates. One single colony was picked and resuspended in Luria-Bertani (LB) broth (244620, Difco Laboratories, Detroit, Mich.) containing streptomycin, tetracycline and ampicillin respectively (LB-STA broth). Same final concentrations of the antibiotics were used as described above (see 3.1.2. *Salmonella* detection by culture). The culture was incubated aerobically at 37 °C in a shaking incubator. After 12 h of growth a subculture was produced by suspending 100 μl of the overnight culture in 2 ml LB-STA broth. This subculture was incubated for 4 h aerobically at 37 °C in a shaking incubator. 500 μl of this subculture was centrifuged and the pellet resolved in 500 μl sterile phosphate-buffered saline (PBS). Each animal received 50 μl of the cell suspension diluted in 150 μl sterile PBS by oral gavage. The administered vaccine dose was determined by serial dilution on LB-STA agar plates and aerobic incubation at 37 °C for 12 h.

3.1.5. Production of the challenge dose

C. jejuni CC21 ST21, from a frozen stock, was streaked onto COL SB agar (PB 5008A, Oxoid, Wesel, Germany) incubated for 24 hours at 42 °C and subcultured twice for 18 h under microaerobic conditions (5 % O_2, 5 % CO_2, 80 % N_2, and 10 % H_2). Two loops of *C. jejuni* CC21 ST21 were picked and suspended in 5 ml sterile PBS. The suspension was diluted to an OD_{600} of 0.43 measured with an Eppendorf BioPhotometer (6131 000.039, Eppendorf, Hamburg, Germany). Each animal received 100 µl of the cell suspension by oral gavage. In order to determine the challenge dose, serial dilutions were plated in duplicates on *Brilliance*™ CampyCount agar plates (PO 5305A, Oxoid) and incubated for 48 h at 42 °C under microaerobic conditions.

3.2. Immunoblot analysis

An overview of reagents used in immunoblot analysis is given in Table 2. Chicken sera, bile and intestinal contents were tested for specific anti *C. jejuni N*-glycan antibodies using "3D5glyc-Bac" and "3D5unglyc" (80 ng/µl loaded of each glycoprotein). The humoral immune response against the *Salmonella* strain was assessed by loading either SKI40 or SKI41 whole cell extracts untreated or proteinase K treated (SKI40pk / SKI41pk) (Table 2). Protein separation was performed with SDS PAGE, proteins were transferred to a polyvinylidenfluorid (PVDF) membrane (IPVH00010, Millipore, Billerica, MA, USA) and blocked in 5 % skim milk for 1 hour at room temperature. Blots were washed 3 times with TTBS (containing 0.1 % Tween20). Chicken sera were diluted 1:100, bile 1:200 and intestinal lavage 1:3 in 1 % skim milk and used in the first incubation step overnight at 4 °C. Blots were washed 3 times with TTBS and then incubated for 2 hours with the secondary antibody. For positive control an anti mouse antibody was used (see Table 2). After washing, blots were developed using enhanced chemilumescent (ECL). Briefly, 1 ml solution A (200 ml 0.1M

TRIS-HCl, 50 mg Luminol) was mixed with 0.3 µl 30 % H_2O_2 and 100 µl solution B (11 mg para-hydroxycoumarin acide in 10 ml dimethyl sulfoxide).

3.3. DNA extraction from swabs and tissue samples

DNA was extracted from caecal contents and cloacal swabs using the QIAamp DNA Stool Mini Kit® (51504, Qiagen, Hilden, Germany) according to the manufacturer's instructions. Caecal content was weighed before DNA extraction to perform a *S.* Typhimurium real-time PCR quantification.

DNA from liver and spleen was extracted with the QIAamp DNA Mini Kit® (250) (51306, Qiagen) following the manufacturer's guidelines.

3.4. Plasmids

DNA products were cloned into a pCR2.1 vector using the TOPO TA cloning kit (45-0641, Invitrogen, Carlsbad, CA, USA) following the manufacturer's guidelines. Plasmid isolation was performed using the QIagen Plasmid Mini Kit (100) (12125, Qiagen, Hilden, Germany). After sequencing (Microsynth, Balgach, Switzerland) the PureYield^{TM} Plasmid Maxiprep System (A2393, Promega, Madsion, WI, USA) was used to increase plasmid DNA. Plasmid concentration and purity was measured using the spectrophotometer "NanoDrop 2000c" (Witec AG, Littau, Switzerland) and diluted in a ten-fold series to establish standard curves for the real-time PCRs. The plasmids were named CJ plasmid, CC plasmid and STM plasmid.

3.5. Real-time PCR

3.5.1. Real-time PCR for the detection of *S.* Typhimurium vaccine strain

The *S.* Typhimurium real-time PCR by Park et al. (2008) was used to detect the *Salmonella* vaccine strain SKI40 and SKI41 in organ samples, caecal contents and cloacal swabs. The Sal-F (5'-GCGCACCTCAACATCTTTC-3') and Sal-R (5'-CGGTCAAATCCACGTTCA-3') primers (Mycrosynth) and the SalP probe (5'-FAM-ATCATCGTCGACATGCMGB/NFQ-3') (Applied Biosystems) amplify a 62 bp fragment of the STM4497 gene from *S.* Typhimurium LT2.

The PCR reaction mixture of a total volume of 25 µl contained: 12.5 µl TaqMan®Universal PCR MasterMix (2x) (10-pack (10x5ml), 4305719**,** Applied Biosystems), 400 nM forward primer Sal-F, 400 nM reverse primer Sal-R, 200 nM of the probe Sal-P and 10 µl extracted sample DNA.

PCR was carried out on a 7500 Real-Time PCR System with Dell™ Notebook (4351104, Applied Biosystems). The threshold value was set to 0.01 with the baseline ranging from 3-15.

Quantitation of the *Salmonella* strains SKI40 and SKI41 in ceacal contents of vaccinated chickens was done according to the standard curve approach (User bulletin #2: Relative quantitation of gene expression, Applied Biosystems).

3.5.2. Duplex real-time PCR for *C. jejuni* and *C. coli*

To exclude *Campylobacter* in feed and litter and to prove that day-of-hatch chickens were free of *Campylobacter*, the *Campylobacter* duplex real-time PCR for the detection of *C. jejuni* and *C. coli* was performed essentially as described by Hong et al. (2007). This duplex real-time PCR was chosen based on its excellent performance in the study by Schnider et al. 2010. For *C. jejuni* a 85 bp fragment of the hippuricase gene (*hipO*-gene, Gen Bank accession

number Z36940) was amplified, and for *C. coli* a 81 bp fragment of the *ceuE* gene, which encodes a lipoprotein (*ceuE*, GenBank accession number X88849) was amplified. The primer pair used for *C. jejuni* detection was: HipO-F (5'-CTGCTTCTTTACTTGTTGTGGCTTT-3') and HipO-R (5'-GCTCCTATGCTTACAACTGCTGAAT-3') (Microsynth), and the probe HipO-P (5'-FAM-CATTGCGAGATACTATGCTTTG-MGBNFQ-3') (Applied Biosystems, Foster City, CA, U.S.A.). The primer pair for *C. coli* was CeuE-F (5'-GATAAAGTTGCAGGAGTTCCAGCTA-3') and CeuE-R (5'-AACTCCACCTATACTAGGCTTGTCT-3') (Microsynth), and the probe Ceu-P (5'-VIC-CTGTAAGTATTTTGGCAAGTTT-MGBNFQ-3') (Applied Biosystems.)

Duplex real-time PCR was performed on a 7500 Real-Time PCR System with Dell™ Notebook (4351104, Applied Biosystems), using the standard DNA thermal cycle protocol. The protocol by Hong et al. (2007) was altered as subsequently described: A total reaction volume of 25 µl was used containing 12.5 µl of TaqMan®Universal PCR MasterMix (2x) (10-pack (10x5ml), 4305719, Applied Biosystems), 300 nM of each primer, 200 nM of each probe, and 7 µl extracted DNA from the sample. The data were analysed with the program 7500 Fast System SDS Software version 1.4.0 (Applied Biosystems). Baseline was set from 3 to 15, with a threshold adjusted to 0.3 for *C. jejuni* and *C. coli*.

3.5.3. Real-time PCR for chicken GAPDH

As an internal positive control (IPC) for proper working of DNA extraction, an additional real-time PCR detecting the chicken glyceraldehyde 3-phosphate dehydrogenase (GAPDH) pseudo gene was performed (Schybli 2010). The PCR reaction mixture contained 12.5 µl TaqMan®Universal PCR MasterMix (2x) (10-pack (10x5ml), Order number: 4305719, Applied Biosystems), 400 nM of forward primer For_GAPDH (3'-GCACGCCATCACTATCTTCCA-5') and reverse primer Rev_GAPDH (3'-TGCCCATTTGATGTTGCTGG-5'), 200 nM of the probe Probe_GAPDH

(3'-HEX-TAGCTTTCTCCTCTTGCCACTCCAGAGGATG-TAMRA-5') and 10 µl extracted DNA. The threshold value was set to 0.01 with the baseline ranging from 3-15.

3.6. Chickens

70 commercial Ross PM3 broiler chickens were obtained from a hatchery (Erb Brüterei AG, Aeschlen bei Oberdiessbach, Switzerland) on the day-of-hatch. The broiler breeders and the hatchery are free of *Salmonella* due to a stringent testing regime by order of the Federal Veterinary Office (*Anonymous*, 2009). The parent breeders had a history of being *Campylobacter*-free, tested every 8 weeks using culture.

Before placing newly hatched chickens into the isolators (390-07-04, LaCalhène, France) they were leg ringed. Cloacal swabs were taken and the *Salmonella* vaccine was administered by gavage. Commercial chicken feed (UFA 513, Alleinfutter für Küken mit Kokzidiostatikum, UFA AG, Sursee, Switzerland) and water were provided *ad libitum*. The box liners, feed, and bedding wood shavings were cultured to exclude *Campylobacter* and *Salmonella* spp. Cloacal swabs were tested by *C. jejuni* / *C. coli* duplex real-time PCR. Animals were weighted daily and at least twice a day checked for clinical symptoms (general condition, diarrhoea, weight loss, ruffled feathers and dyspnoea).

3.7. Animal experiments

3.7.1 Experiment 1.1

14 one-day old chickens were randomly divided into two equal groups and vaccinated by gavage (Table 1). Group 1A received 2.5×10^7 cfu of SKI40 vaccine strain expressing the *Campylobacter N*-glycan. Group 1B was vaccinated with a dose of 3×10^7 cfu of the *Salmonella* SKI41 strain which served as a "backbone" control. Blood was taken from the wing vein on day 10, 15 and 20. On day 25 all birds were killed and dissected. Quantitative

bacteriology and plasmid stability of the *Salmonella* vaccine strain was performed with caecal contents of 4 animals from each group and confirmed by semi-quantitative *Salmonella* real-time PCR. Bile stored at -20 °C.

1 g of intestinal contents from the ileum was mixed in 10 ml protease inhibitor cocktail cOmplete (04 693 116 001, provided in *EASY*pack Roche, Rotkreuz, Switzerland). After shaking and centrifugation at 10,000 x g for 30 min at 4 °C, the supernatant containing the sIgA was collected and stored at -20 °C.

3.7.2. Experiment 1.2

Immunoblot analysis was performed with sera from groups 1A and 1B and with pooled pre-immune sera (see below). The anti *N*-glycan IgY antibodies in the sera were checked against truncated forms of the *Campylobacter* heptasaccharide by immunoblotting. An overview of the different truncated oligosaccharides is given in Table 2.

3.7.3. Experiment 2

For challenge experiment, chickens were divided into 4 groups (2A-2D) with 14 chickens each. 10^7 cfu of the SKI40 vaccine strain was applied to group 2A. Group 2B served as negative control and received sterile PBS at day-of-hatch. Group 2C received 5 x 10^7 cfu of SKI40 at the day-of-hatch and additionally a booster dose, five days later, containing 1.25 x 10^7 cfu SKI40. In the backbone control (group 2D) each animal received 4 x 10^7 cfu SKI41 on day one of age and 1.35 x 10^7 cfu SKI41 on day 5 by gavage. Chickens from group 2A and 2B were challenged at day 14 with 3 x 10^8 cfu *C. jejuni* CC21 ST21, and groups 2C and 2D with 5.25 x 10^7 cfu respectively. During the experiments three chickens of each group were killed and dissected on day 18, 23, and 28, and five at the end. Additionally blood was taken from the wing vein on day 10, 15, 20, 25 and 30. As described above qualitative bacteriology and *S.* Typhimurium real-time PCR was performed with liver and spleen.

Colony counts per gram caecal contents of the vaccine strain SKI40 and SKI41 and the challenge strain *C. jejuni* CC21 ST21 was performed using culture. Sera were used for immunoblot analysis and screened for a specific humoral immune response. Bile and intestinal contents were also collected to detect sIgA.

To check the *C. jejuni* CC21 ST21 stability at the end of the experiment, the challenge strain was isolated from caecal contents and typed as described by Wirz et al. (2010). To detect the amount of the *N*-glycan on the *Salmonella* surface of SKI40 recovered from caecal contents, immunoblot analysis was performed using the polyclonal anti *N*-glycan rabbit serum named hR6 (Table 2).

For all experimental groups, pre-immune serum was obtained and pooled from day-of-hatch chicken from the same batch.

3.8. Statistics

Statistical analysis was performed using the exact Mann-Whitney U Test (Prism_5). $P < 0.05$ (two-tailed) was considered to be statistically significant.

4. Results

4.1. Standard curve analysis of the *S.* Typhimurium, *C. jejuni* and *C. coli* real-time PCR

Linearity persisted over the range of 10^5 copies to 10^1 copies for *S.* Typhimurium**,** 10^5 copies to 10^1 copies for *C. jejuni*, and 10^4 copies to 10^0 copies for *C. coli*. The coefficients of determination for the linear regression were: 0.9959, 0.9972 and 0.9992, respectively (data not shown).

4.2. Animal experiments

In all animal experiments feed, bedding and box liners were free from *Salmonella* and *Campylobacter*. Cloacal swabs of all one-day-old chickens were negative by *C. jejuni* / *C. coli* duplex real-time PCR.

4.2.1. Experiment 1.1

Self-limiting diarrhoea was observed from day 2 to 5 in all vaccinated groups. However, none of the chickens showed loss of appetite, ruffled feathers, weight loss, or other health problems. Upon necropsy, the vaccine as well as the backbone control were detected by culture and real-time PCR in liver, spleen and in caecal contents of 14 animals from groups 1A and 1B ($8 \times 10^5 - 3 \times 10^7$ cfu/g caecal contents and $6 \times 10^5 - 10^7$ cfu/g, respectively) (Table 3).

Plasmid stability in the vaccine and backbone control was demonstrated (Table 1). A high plasmid stability can be deduced in SKI41, $8.7 \times 10^6 - 4.57 \times 10^7$ SKI41 cfu per gram caecal contents were still carrying the pBR322 plasmid after 25 days (Figure 1). Until the end of the experiment, the vaccine *Salmonella* SKI40 still harboured the pBR*pgl*3mut. However, the spread between plasmid carrying and non-carrying bacteria was pronounced, ranging from 7×10^3 to 1.7×10^7 SKI40 cfu/g caecal contents (Figure 1).

Humoral immune response against the *S.* Typhimurium vector strain could be detected in all sera from chickens of groups 1A and 1B (Figure 2A and 2B). IgY against the *Campylobacter N*-glycan was present in all sera with a marked decrease over time (Figure 2A). Pooled pre-immune sera from day-of-hatch chickens also showed specific IgY against *Campylobacter N*-glycan (data not shown). No anti heptasaccharide IgM antibodies were detected in immunoblots until day 25 in the vaccinated group 1A (Figure 2B).

4.2.2. Experiment 1.2

In the immunoblot analysis full-length and truncated forms of the *Campylobacter* heptasaccharide (Table 2) were tested as antigens to analyse substrate specificity of IgY. In Figure 3A, IgY in sera from one animal reacting with entire and truncated oligosaccharides is shown at different time points. hR6, used as a positive control (Table 2), did recognize all glycan structures but not the truncated Bac-GalNAc (Figure 3B). The anti *N*-glycan IgY in sera from the chickens showed the same binding pattern as hR6 (Figure 3A and Figure 3B).

4.2.3. Experiment 2

No significant reduction of the *C. jejuni* CC21 ST21 challenge strain was detected in vaccinated compared to unvaccinated broiler chickens by quantitative culture analysis, with the exception of a significant reduction of the caecal *C. jejuni* load between group 2A (vaccine) and group 2B (PBS) at day 35. Anti *N*-glycan IgY antibodies were present in the pre-immune sera (data not shown) and in sera of 4 animals out of 5 from group 2C (vaccine) and 2D (backbone) (Table 4) until day 15. Two animals from group 2C and three animals of group 2D showed anti *N*-glycan IgY on day 35 (Table 4 and Figure 4A). In all challenged groups, anti heptasaccharide IgM (Table 4) in serum and sIgA in intestine and bile was detected on day 35 (Figure 4B and 4C). *C. jejuni* isolated from caecal contents at day 35 was

typed and confirmed to be *C. jejuni* CC21 ST21. Likewise, the SKI40 vaccine strain was confirmed to be carrying the *pgl*3mut operon using hR6 in immunoblot analysis.

5. Discussion

Poultry meat consumption is a known risk factor for human campylobacteriosis (Harris et al. 1986). Contamination due to *Campylobacter* on the carcasses during processing at slaughter varies between 2 $\log_{10}$-6 $\log_{10}$ cfu (Jacobs-Reitsma et al. 2008). A risk-based modelling on human campylobacteriosis calculated the requested decrease in the *Campylobacter* load in intestine of chickens, to achieve reduction of human cases (EFSA 2011). Briefly, the EFSA declared that each $\log_{10}$ reduction of the *Campylobacter* load in chicken intestine would mean a 0.64 $\log_{10}$ decrease on the meat. This reduction of the *C. jejuni* in the chicken intestine would result in a campylobacteriosis related public health risk reduction of 48 %. Reaching a 2 $\log_{10}$ or 3 $\log_{10}$ intestinal load reduction would even reduce the risk of human campylobacteriosis by a 76 % or 90 % reduction (EFSA 2011).

Vaccination against *Campylobacter* is one strategy to achieve a reduction of the caecal load in chickens. Vaccine strategies to immunize broiler chickens against *Campylobacter* have already been explored in different studies (De Zoete et al. 2007, Rice et al. 1997, Wyszynska et al. 2004, Buckley et al 2010, Layton et al. 2011), but *Campylobacter* vaccines are still in the development phase and most published studies had a poor reproducibility (EFSA 2011).

Commensal bacteria and the host immune system interact in the way that sIgA is produced in the gut in response to the bacterium in the lumen, with sIgA preventing the bacteria from becoming invasive. Bacteremia readily induces a serum IgG response, while no IgA can be found in the serum (Macpherson et al. 2000). Concurrent with this knowledge about commensals, *Campylobacter* in chickens is known to dwell in the lumen, mucus and intestinal crypts, but does not invade the gut wall. In contrast to intestinal inflammation in campylobacteriosis in humans, *Campylobacter* does not elicit inflammatory responses in chickens (De Zoete et al. 2007).

An oral vaccine should be able to induce first line sIgA response, but also immunology memory. Gut mucosal vaccines that are used to prevent human diseases by enteric pathogens

generally have mixed success (Pasetti et al. 2011). Oral polio vaccination was as success story with a worldwide 99 % decrease in cases since 1988 (WHO 2011a). Vaccines against typhoid fever, however, gave different results in different geographic regions. While the oral liquid formulation of *S.*-Typhi Ty21a vaccine was shown to be 77 % effective over 5 years in a trial in Chilean school children (Levine et al. 1999, WHO 2011b), the vaccine only showed 33-53 % protective efficacy in a trial in Indonesia (WHO 2011b). The immunologic basis for these differences remains elusive, mostly because the mechanisms of the gut immune system are only partially understood (Pasetti et al. 2011). When an enteric pathogen invades the human gut, the innate immune system anneals and processes the antigens and, depending on the agent, secretes pro-inflammatory signals. The adaptive immune system is responsible for specific antigen recognition, specific effector functions and the immunologic memory (Pasetti et al. 2011). Cells involved in antigen capture, processing and presentation are foremost M-cells and dendritic cells, while B-lymphocytes and T-lymphocytes secret antibodies, cytotoxins and fulfil cytotoxic functions (Pasetti et al. 2011). It was shown in humans that oral vaccines can elicit a local mucosal sIgA response to prevent attachment and invasion. They can neutralise enterotoxins and also induce systemic serum IgG against invasive agents. Further cell-mediated immune response and antibody-dependent cellular toxicity response is stimulated (Pasetti et al. 2011).

B-cells that have been primed with the vaccine antigen migrate into the gut-associated lymphoid tissue and / or local lymph nodes and subsequently multiply and differentiate. IgA+ plasmablasts derived from these B-cells differentiate into polymeric IgA-secreting plasma cells in the lamina propria where they secret sIgA. This last differentiation step is mediated by activated CD4+ T-helper 2 cells (interleukin 2 (IL-2), IL-5, IL-10), dendritic cells (retinoic acid, IL-10, transforming growth factor β (TGF-β), and intestinal epithelial cells (TGF-β, IL-6) (Pasetti et al. 2011).

Kaiser et al. (2009) stated that one has to be prudent to extrapolate research in the mammalian immune system to the avian immune system. In T-cell deficient mice, B-1 cells have been shown to be able to produce sIgA in response to commensal bacteria when interacting with dendritic cells (Macpherson et al. 2000). The implication of this finding for birds is, however, unknown, as birds may altogether use other ways to generate such a response e.g. via the bursal lumen (Muir et al. 2000). Chickens clearly have their own cellular and humoral immune response and are simply not "feathered mice" (Kaiser et al. 2009)

In chickens, sIgA are the first defence of the mucosal epithelia against enteric pathogens (Muir et al. 2000, De Zoete et al. 2007). The chicken intestinal immune system does have an antigen-specific sIgA antibody response against enteric pathogens that inhibits attachment and colonization of the pathogen. Unfortunately, the innate immune response and the immune cells and their secretions are poorly understood in the avian host and fundamental work in this area remains to be done (Kaiser et al. 2009). Secretion of intestinal sIgA in the chicken against antigens presented in the intestinal surface from *Salmonella* Typhimurium or *Campylobacter* was demonstrated in different studies (Muir et al. 1998, Noor et al. 1995, Widders et al 1996). Nonetheless, vaccines to control intestinal pathogens in chickens do not have the expected success (Muir et al. 2000).

Increase of *C. jejuni* specific antibodies in chicken and the resulting reduction of bacterial shedding was observed during colonization, suggesting an effective immune response against these bacteria (De Zoete et al. 2007). In contrast to human infection, chickens however show no inflammatory response and tissue damage. Two to three weeks after experimental inoculation with *Campylobacter*, specific serum IgY, IgA and IgM are detectable. sIgA from bile and intestine are present at week 3 to 4 (De Zoete et al. 2007).

In this study we investigated the potential of a *S.* Typhimurium vaccine strain presenting a *Campylobacter N*-glycan on its surface.

As it has been shown that oral and intramuscular infection with *S*. Typhimurium in different inbred chicken lines at day-of-hatch show strain-dependent mortality of 0 % to up to 100 % (Bumstead and Barrow 1988), we investigated attenuation of the *S*. Typhimurium vector strain in a first experiment. Sufficient attenuation of the human-derived vaccine strain SKI40 and SKI41 orally administered to untreated commercial day-of-hatch chicks could be demonstrated. The chicks developed a self-limiting diarrhoea from day 2 until day 5 *p.i.*. The bedding was therefore more moist, which may be a problem at a large scale. Upon necropsy, *Salmonella* was detected in liver, spleen and caecal content by culture and real-time PCR. Concurrence between culture and real-time PCR results was good. Minor discrepancies can be explained with the fact that two different pieces of organs had to be used for culture and PCR quantification. Furthermore, faeces is a difficult matrix for real-time PCR analysis. Different to carcass rinse, where real-time PCR was always found to be more sensitive than culture (Botteldoorn et al. 2008, Schnider et al. 2010), this is not necessarily the case in faeces (Inglis and Kalischuk 2004). Groups 1A and 1B had 8 x 10^5 to 3 x 10^7 cfu Salmonellae per g caecal contents harvested at day 25. Chicks received 2.5 x 10^7 to 3 x 10^7 cfu by oral gavage, thus it can be deduced that the bacteria were able to colonize the caeca. Immune reaction against various proteins and ECA of the SKI40 whole cell extract was demonstrated in all inoculated chickens (Figure 2, lane 1).

Although parent breeders of all experiment chickens had a history of being free of *Campylobacter* (tested by faecal bacteriology), specific anti *N*-glycan IgY were found in pooled pre-immune sera. It was observed, that the amount of anti *N*-glycan IgY antibodies in all groups markedly decreased over time from day-of-hatch to day 25 (Figure 2A and 4A). Thus, we conclude, that maternal anti *Campylobacter* antibodies were present in the chickens. The occurrence of specific maternal anti *Campylobacter* antibodies in newly hatched chickens is well known and suspected to prevent or at least inhibit natural infection in the first 10 days

of the so-called lag-phase (Newell and Fearnley 2003). Maternal IgY are decreasing over the first two to three weeks (Myszewski and Stern 1990, Cawthraw et al. 1994).

According to current knowledge, Bac-GalNAc$_5$-Gluc only exists in *Campylobacter* and not in other bacteria. To prove specificity of IgY detected in large amounts in pre-immune sera and thereafter diminishing over time, we tested different entire and truncated forms of the *Campylobacter* heptasaccharide. A decline of maternal IgY binding to all truncated glycoproteins is seen (Figure 3A) with exception of the truncated Bac-GalNAc. The positive control serum hR6 (produced in rabbits vaccinated with *Campylobacter N*-glycan expressing *E. coli*) (Figure 3B) and all sera from groups 1A and 1B yielded the same reactivity. Consequently, bacillosamine can be excluded as the crucial immunogenic part, because sera from groups 1A and 1B did not react with Bac-GalNAc and further reacted with the engineered glycan containing GlucNAc instead of bacillosamine.

Thus, although the parent breeders have a history of being *Campylobacter*-free, the sera of the chicks showed maternal IgY against the *N*-glycan until day 25. This unexpected result discloses the testing regime of the breeders to be insufficient to prove *Campylobacter*-freedom. Alternatively, the birds were able to completely clear the bacteria (Achen et al. 1998, Newell and Fearnley 2003).

The *C. jejuni* CC21 ST21 used in the challenge experiments was isolated both from caeca and neck skin from a slaughter batch of Swiss broilers (Wirz et al. 2010). This strain was chosen as it derived from a naturally infected flock and that 18.5 % of poultry-derived *C. jejuni* belonged to CC21 (which comprised of ST 19, 21, 50, 262, 883, 917 and 3988) and thereof 7.0% to ST 21, rendering those complexes and/or types the predominant in a recent Swiss study. Furthermore CC21 is one of the most frequently reported clonal complex in human campylobacteriosis (Wirz et al. 2010, Hermans et al 2011).

Figure 4 shows that the challenge with *C. jejuni* provoked a specific humoral immune response against the *Campylobacter N*-glycan in a chicken in group 2C. Likewise, it could be detected in all challenged groups (Table 4). Anti heptasaccharide IgM were present in sera at day 30. Approximately 20 days after challenge, IgY in sera, sIgA in intestinal lavage and in bile were detectable (Figure 4B and 4C). This is in accordance with Baqar et al. (1995), who used a *Campylobacter* killed whole-cell vaccine, and detected specific IgG antibodies at day 20 *p.i.* in mice. The vaccine study performed by Wyszynska et al. (2004), using a *S.* Typhimurium expressing the CjaA protein of *C. jejuni*, showed specific anti CjaA IgY rising from week 6 with a peak at week 8 after vaccination in chickens. Despite this late specific humoral immune response, the vaccine was shown to reduce the ability of the *Campylobacter* challenge strain to colonize the intestinal tract of the chicken. However, with an infective dose of 500 to 800 cfu in humans (Janssen et al. 2008), and a caecal burden of up to 10^9 bacteria per g faeces in chickens (Newell and Fearnley 2003), reduction would have to be substantial. In our challenge experiments, using a similar vaccination strategy as Wyszynska et al. (2004) (vaccinating on day-of-hatch, use of booster), no significant reduction of the *C. jejuni* caecal load was achieved due to vaccination with the exception of a significant reduction of the caecal *C. jejuni* load between group 2A (vaccine) and group 2B (PBS) at day 35 (Figure 5). The *Campylobacter N*-glycan was nonetheless shown to be immunogenic in chicken. Disrupted *N*-glycosylation in *C. jejuni* was demonstrated to result in impaired colonization of chicken intestinal tract (Szymanski et al. 2002, Karlyshev et al. 2004), and thus selected as a potent antigen for a *Campylobacter* vaccine. Moreover, the heptasaccharide is conserved among *Campylobacter jejuni* and *coli* (Szymanski and Wren 2005). However, the immune response shown in the chickens in this study only produced a specific immune response against naturally presented *C. jejuni N*-glycan linked to different proteins in *Campylobacter*, and not against the engineered *C. jejuni N*-glycan linked to the lipid A core of the *Salmonella* vaccine strain. It currently remains elusive, why this vaccine

strain could not induce immune reaction against its inbuilt *C. jejuni N*-glycan. However, all immunized chicken reacted against the *Salmonella*. Further studies need to assess, whether the vaccination protocol should be altered (time point of first inoculation and boosters), or whether the antigen presentation is adequate, i.e. whether the vaccine *Salmonella* needs to present the *C. jejuni N*-glycan on proteins or the *C. jejuni N*-glycan is not present in sufficient quantity on the *Salmonella* surface

6. Conclusion

The new *S.* Typhimurium vaccine strain SKI40, expressing the engineered *Campylobacter N*-glycan Bac-GalNAc$_5$-Glc, was shown to be attenuated sufficiently for oral vaccination of commercial day-of-hatch broiler chicks and the O-antigen deficient vaccine strain was able to colonize the caeca of the chickens. Vaccination of chickens did not lead to a significant reduction of *C. jejuni* colonization in chicken caeca with the herein used antigen presentation and / or vaccination protocol. In immunoblot analysis no specific humoral IgM immune response against the *C. jejuni N*-glycan was found after vaccination. However, in the challenge experiments IgM, IgY and sIgA against the *Campylobacter N*-glycan were demonstrated after challenge in the vaccinated groups as well as in the control groups. This specific immune response is due to *Campylobacter* challenge on day 14, and not due to vaccination. In all sera maternal anti *N*-glycan IgY antibodies were detectable and declined over time until day 25. In a trial with several truncated *Campylobacter* glycans, the minimal antigenic structure was defined to be Bac-GalNAc$_2$.

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8. Figures and Tables

Table 1. Bacterial strains and plasmids.[1]

Bacterial strains	**Used in / for**	**Source or reference**
SKI40 = *Salmonella enterica* subsp. *enterica* serovar Typhimurium *SL1344*Δ*wbaP*, integration of pBR*pgl3*mut (engineered *Campylobacter N*-glycan with a GlucNAc instead of the bacillosamine) Antibiotic resistance on genome: S, T	animal experiments	WO 2010/108682 A1 "*Salmonella enterica* presenting *C. jejuni N*-glycan or derivatives thereof"
SKI41 = *Salmonella enterica* subsp. *enterica* serovar Typhimurium *SL1344*Δ*wbaP*, integration of pBR322 (backbone without *Campylobacter N*-glycan) Antibiotic resistance on genome: S, T	animal experiments	WO 2010/108682 A1 "*Salmonella enterica* presenting *C. jejuni N*-glycan or derivatives thereof"
Campylobacter jejuni / *Campylobacter coli* field strains	real-time PCR	confirmed Swiss field isolates IVB, University of Zürich, CH
Campylobacter jejuni CC21 ST21 (Ue1655)	animal experiments, challenge strain	IVB, University of Bern / Wirz et al. 2010
Plasmids	**Used in / for**	**Source or reference**
pBR322 Antibiotic resistance on plasmid: A	animal experiments	Karin Ilg, Department of Microbiology, FIT, CH
pBR*pgl*3mut Antibiotic resistance on plasmid: A	animal experiments	Karin Ilg, Department of Microbiology, FIT, CH

[1]Abbreviations (in alphabetical order): A = ampicillin, CC = clonal complex, CH = Switzerland, CRL = community reference laboratory, FIT = federal institute of technology, IVB = institute of veterinary bacteriology, NL = The Netherlands, PCR = polymerase chain reaction, S = streptomycin, ST = sequence type, T = tetracycline

Table 2. Serum, antibody, protein, glycoprotein and bacterial extract.[1]

	Abbreviation used in manuscript	Used for	Reference / obtained from
Serum			
rabbit anti *C. jejuni N*-glycan serum	hR6	positive control IB (diluted 1:5000)	Amber 2008, FIT
Antibody			
polyclonal goat anti-mouse IgG HRP conjugated		2nd antibody IB	Catalog Number: sc-2005, Santa Cruz Biotechnology Inc., (Santa Cruz, CA, USA)
goat anti-rabbit IgG antibody HRP conjugated		2nd antibody IB	Catalog Number: sc-2004, Santa Cruz Biotechnology Inc., (Santa Cruz, CA, USA)
goat anti-chicken IgG (IgY)-Fc antibody HRP conjugated		2nd antibody IB	Catalog Number: A30-104P, Bethyl Laboratories, Inc. (Montgomery, TX, USA)
goat anti-chicken IgM antibody HRP conjugated		2nd antibody IB	Catalog Number: A30-102P, Bethyl Laboratories, Inc.
goat anti-chicken IgA antibody HRP conjugated		2nd antibody IB	Catalog Number: A30-103P Bethyl Laboratories, Inc.
Protein			
murine scFv of the anti-His tag antibody 3D5	3D5unglyc	IB	Lizak et al. 2011
Glycoprotein			
3D5-BacGalNAc$_5$-Glc, naturally occurring *Campylobacter N*-glycan	3D5glyc-Bac	IB	Karin Ilg, Department of Microbiology, FIT
3D5-GlcNAc-GalNAc$_5$-Glc, glycan expressed on *S.* Typhimurium SKI40	3D5glyc-GlcNAc	IB	Karin Ilg, Department of Microbiology, FIT
3D5-Bac-GalNAc$_5$ (truncated form)		IB	Karin Ilg, Department of Microbiology, FIT
3D5-Bac-GalNAc$_2$ (truncated form)		IB	Karin Ilg, Department of Microbiology, FIT
3D5-Bac-GalNAc (truncated form)		IB	Karin Ilg, Department of Microbiology, FIT
Bacterial extracts			
whole cell extracts of SKI40 and SKI41	SKI40 and SKI41	IB	Karin Ilg, Department of Microbiology, FIT
proteinase K treated whole cell extracts of SKI40 and SKI41	SKI40pk and SKI41pk	IB	Karin Ilg, Department of Microbiology, FIT

[1]Abbreviations (in alphabetical order): Bac = bacillosamine, FIT = federal institute of technology, GalNAc = *N*-Acetylgalactosamine , GlcNac = *N*-Actelylglucosamine, His = polyhistidine, HR = horseradish peroxidase, IB = immunoblot analysis, Ig = immunoglobulin

Table 3. Detection of *Salmonella* Typhimurium vaccine strain in liver, spleen and caecal contents of broiler chickens 25 days after oral vaccination with 2.5 x 10^7 cfu SKI40 (group 1A) 3 x 10^7 cfu SKI41 (group 1B).[1]

	monitored by culture			**monitored by real-time PCR**		
	liver	spleen	caecal contents	liver	spleen	caecal contents
	positive/total		cfu/g	positive/total		cfu/g
Group 1A (SKI40)	6/7	2/7	8 x 10^5–3 x 10^7	5/7	2/7	6 x 10^5-1 x 10^7
Group 1B (SKI41)	4/7	3/7		4/7	1/7	

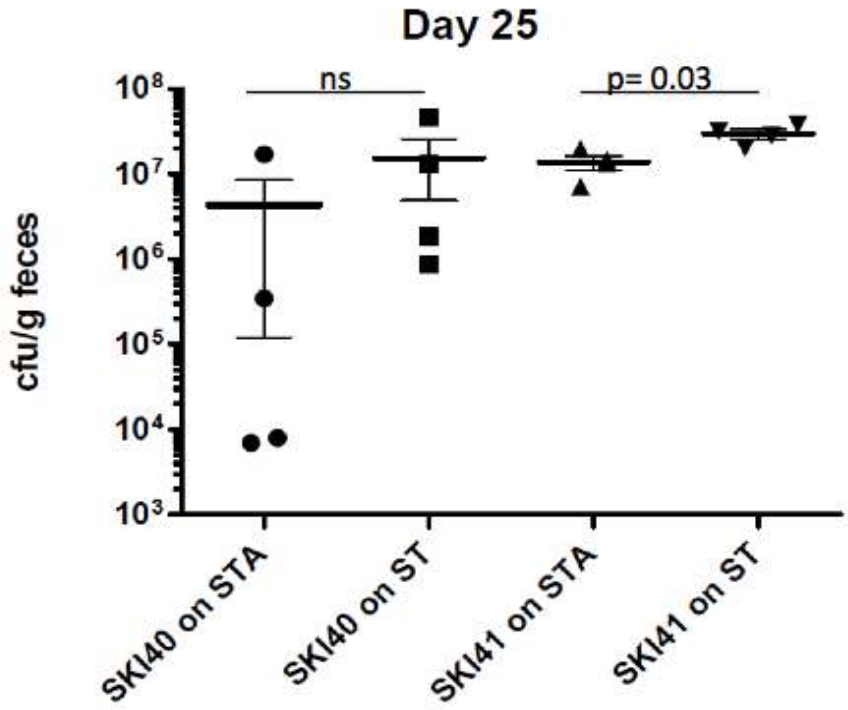
[1]Abbreviations (in alphabetical order): cfu = colony forming units, PCR = polymerase chain reaction, SKI40 = vaccine *Salmonella*, expressing the *Campylobacter N*-glycan, SKI41 = backbone control, *S.* Typhimurium = *Salmonella enterica* subsp. *enterica* serovar Typhimurium

Table 4. Anti *Campylobacter N*-glycan IgY and IgM kinetics in sera from groups 2A (SKI40 at day 1), group 2B (PBS at day 1), group 2C (SKI40 at days 1 and 5), and group 2D (SKI41 at days 1 and 5). All groups were challenged at day 14 with *Campylobacter jejuni* strain CC21 ST21. Blood was taken on day 10, 15, 20, 25, 30 and 35.[1]

		Sera with anti-glycan IgY (positive/total)						**Sera with anti-glycan IgM (positive/total)**					
Group	**d**	**10**	**15**	**20**	**25**	**30**	**35**	**10**	**15**	**20**	**25**	**30**	**35**
2A		nd	nd	nd	nd	nd	nd	0/5	0/5	0/5	0/5	0/5	4/5
2B		nd	nd	nd	nd	nd	nd	0/5	0/5	0/5	0/5	1/5	2/5
2C		4/5	4/5	0/5	0/5	1/5	2/5	0/5	0/5	0/5	0/5	3/5	4/5
2D		4/5	2/5	0/5	0/5	1/5	3/5	0/5	0/5	0/5	4/5	5/5	5/5

[1]Abbreviations (in alphabetical order): CC = clonal complex, Ig = immunoglobulin, nd = not done, PBS = phosphate buffered saline, SKI40 = vaccine *Salmonella*, expressing the *Campylobacter N*-glycan, SKI41 = backbone control, ST = sequence type

Figure 1. Stability of the pBR*pgl*3mut plasmid in *S.* Typhimurium SKI40 and of the pBR322 plasmid in *S.* Typhimurium SKI41: recovery from caecal contents on LB agar containing antibiotics, 25 days after oral gavage.[1]



[1]Abbreviations (in alphabetical order): cfu = colony forming units, ns = not significant, SKI40 = vaccine *Salmonella*, expressing the *Campylobacter N*-glycan, SKI41 = backbone control, STA = Luria Bertani agar plates containing streptomycin, tetracycline and ampicillin, ST = Luria Bertani agar plates with streptomycin and tetracycline

Figure 2: Immunoblot analysis using serum from a broiler chicken of group 1A, vaccinated with *Salmonella* Typhimurium vaccine SKI40 expressing the *Campylobacter N*-glycan. Sera were taken on day 10 (a), 15 (b), 20 (c) and 25 (d).

A IgY detection. **B** IgM detection.

Reaction of the sera are shown against *Salmonella* Typhimurium SKI40 whole cell extract (lane 1), lipids and glycolipids of SKI40 after proteinase K treatment (lane 2), unglycosylated 3D5 protein (lane 3) and glycosylated 3D5 protein (Bac-GalNAc$_5$-Glc, lane 4). The control (e) shows 3D5 unglyc (lane 3) and 3D5 glyc-Bac protein (lane 4), with a size of approximately 37 kDa. Positions of size markers are indicated on the left.

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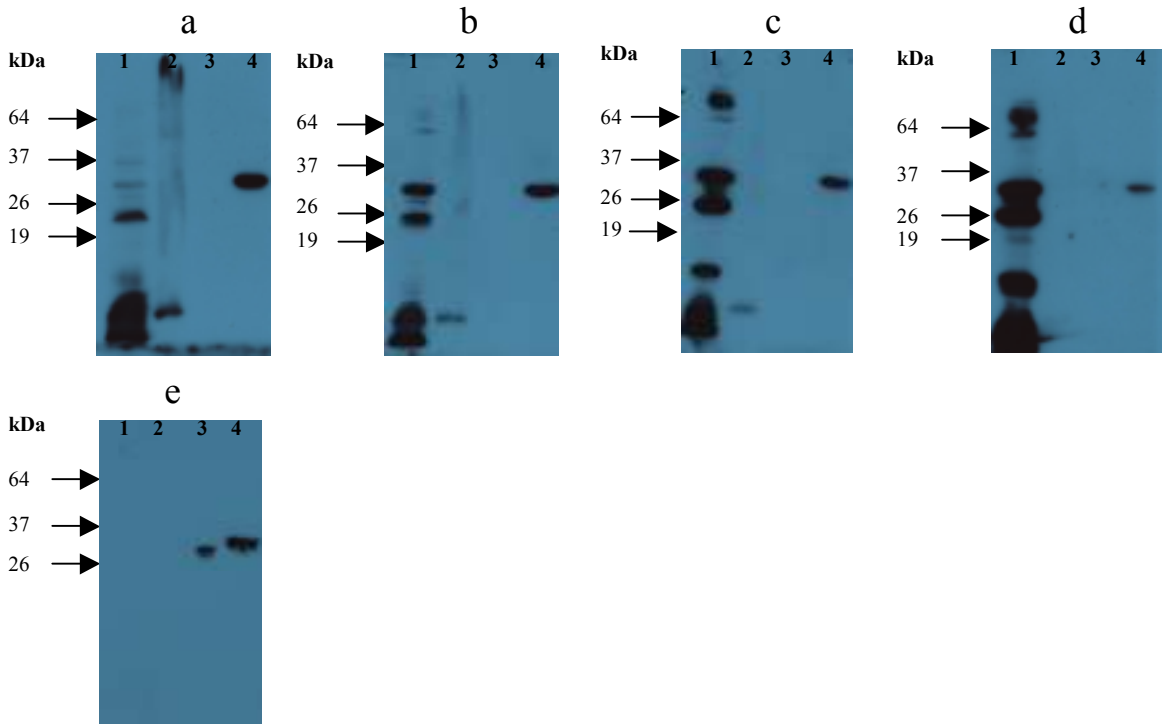

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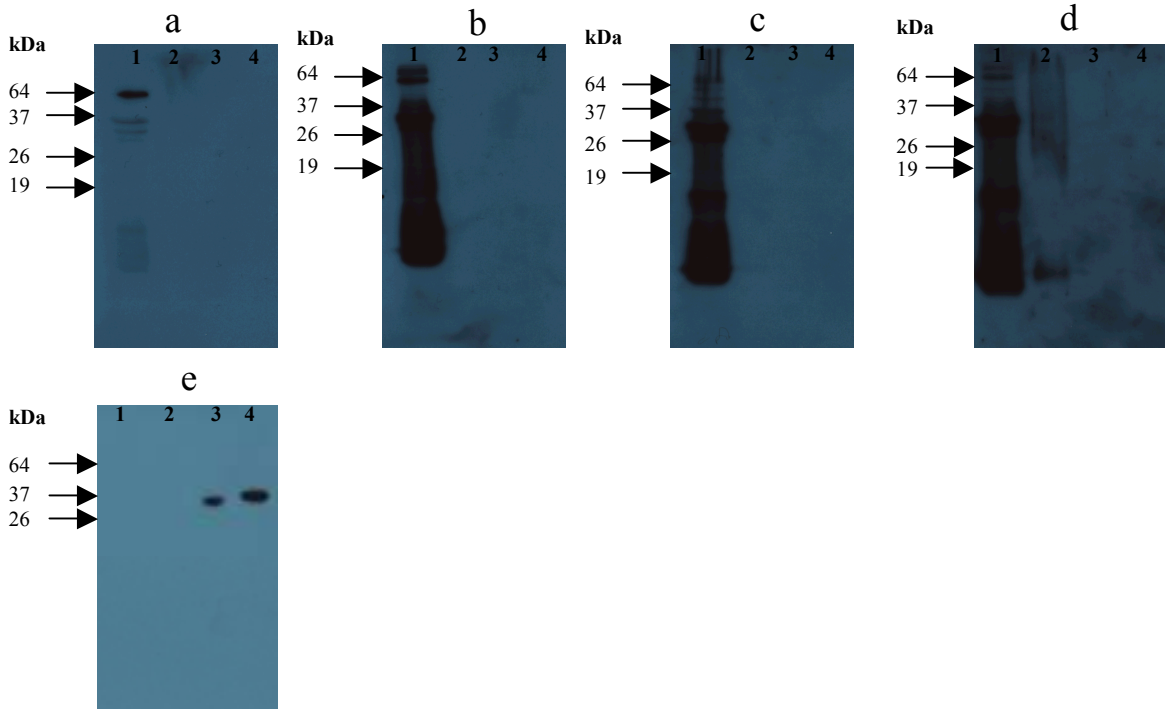
a
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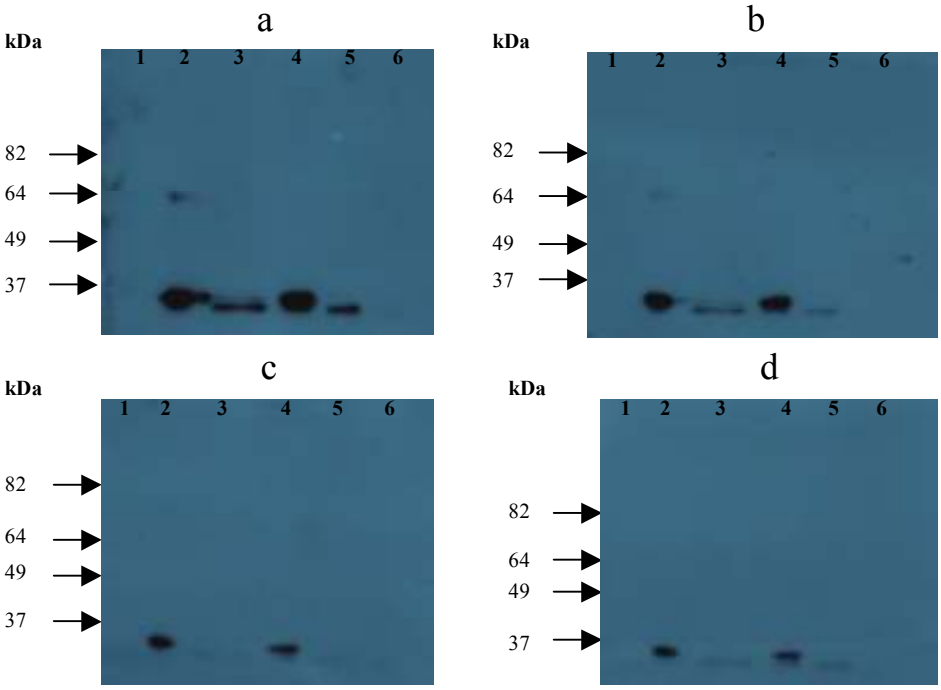
Figure 3: Immunoblot analysis using serum from a broiler chicken of group 1B, vaccinated with the *Salmonella* Typhimurium backbone strain SKI41. Sera were taken on day 10 (a), 15 (b), 20 (c) and 25 (d).

A Exclusion of unspecific IgY in sera, using 3D5 protein carrying truncated forms of the *Campylobacter N*-glycan. **B** Controls for glycan and protein detection, anti *N*-glycan rabbit serum hR6 (e) and polyclonal goat anti-mouse IgG (anti 3D5 carrier protein) (f).

Reaction of the sera against unglycosylated 3D5 protein (lane 1), and the different truncated glycans, 3D5-Bac-GalNAc$_5$-Gluc (2), 3D5-GlcNAc-GalNAc$_5$-Gluc (3), 3D5-Bac-GalNAc$_5$ (4), 3D5-BacGalNAc$_2$ (5) and 3D5-BacGalNAc (6). Positions of size markers are indicated on the left.

A:



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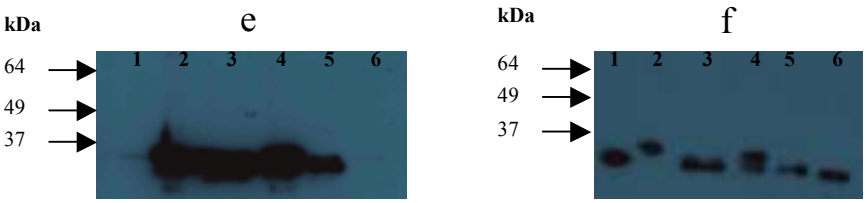
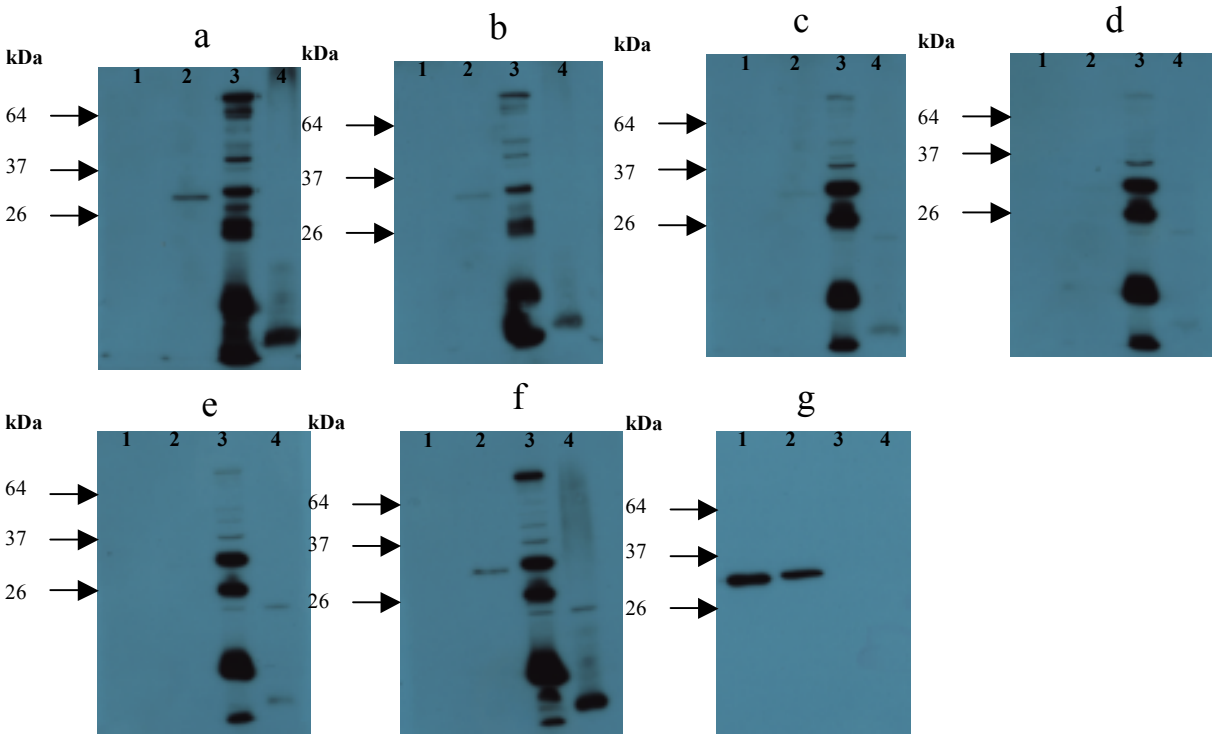


Figure 4. Immunoblot analysis using serum from a broiler chicken of group 2C, vaccinated and boostered with the *Salmonella* Typhimurium vaccine SKI40 expressing the *Campylobacter N*-glycan, and challenged with *Campylobacter jejuni* at day 14. Sera were taken on day 10 (a), 15 (b), 20 (c), 25 (d), 30 (e) and 35 (f).

A IgY detection. **B** IgM detection. **C** Secretory IgA detection in intestinal contents (a) and bile (b) collected at day 35.

Reaction of the sera, intestinal contents and bile are shown against unglycosylated 3D5 protein (lane 1), glycosylated 3D5 protein (Bac-GalNAc$_5$-Glc, lane 2), *Salmonella* Typhimurium SKI40 whole cell extract (lane 3) and lipids and glycolipids of SKI40 after proteinase K treatment (lane 4). The controls (**A** g, **B** g and **C** c) show 3D5 unglyc (lane 1) and 3D5 glyc-Bac protein (lane 2), with a size of approximately 37 kDa. Positions of size markers are indicated on the left.

A:



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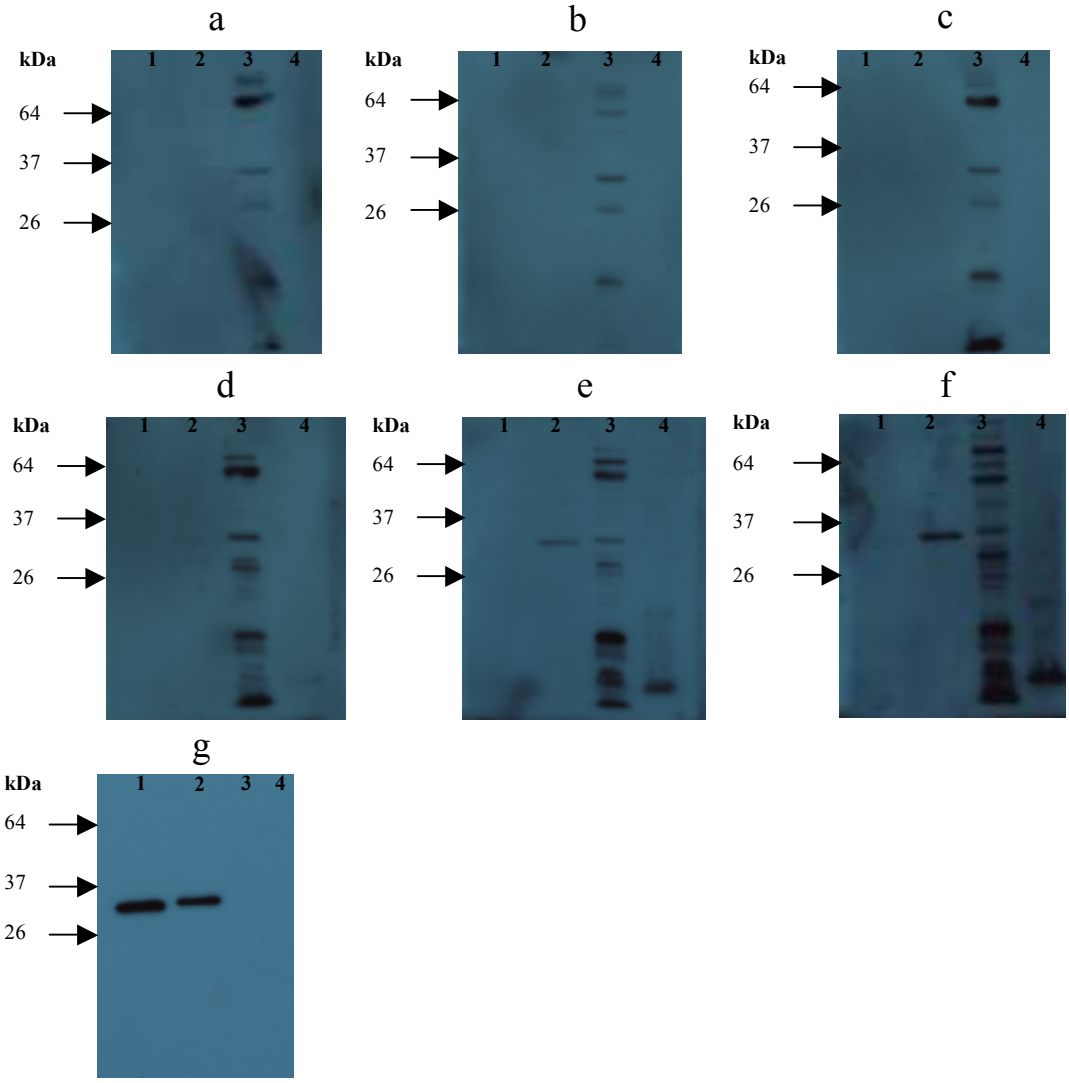


C:

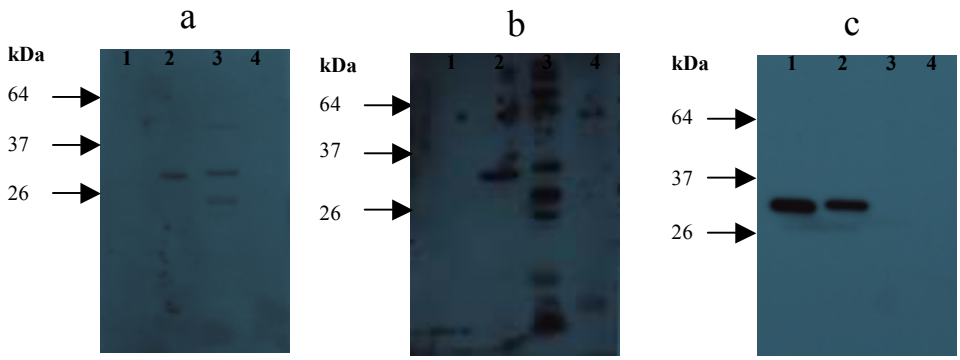
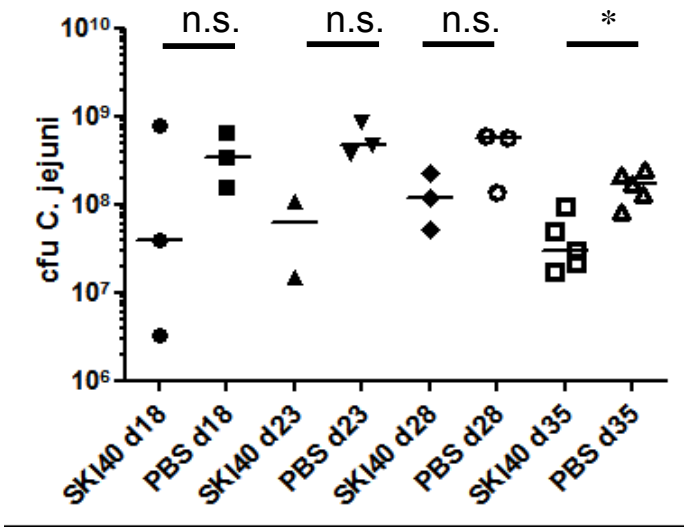


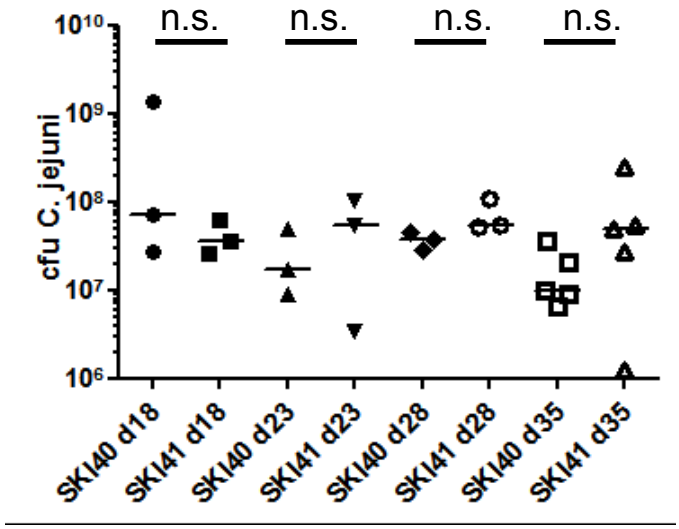
Figure 5. *Campylobacter jejuni* (cfu/g) in caecal contents at different time-points after challenge. Groups 2A and 2B were inoculated with the vaccine strain SKI40 or PBS on day 1 and challenged on day 14 with 3 x 10^8 cfu *C. jejuni*. Groups 2C and 2D were inoculated with the vaccine SKI40 or the backbone control SKI41 on day 1 and 5 and challenged at day 14 with 5.25 x 10^7 cfu *C. jejuni*.[1]

A Groups 2A and 2B. **B** Groups 2C and 2D.

A:



B:



[1]Abbreviations (in alphabetical order): cfu = colony forming units, n.s. = not significant, SKI40 = vaccine *Salmonella*, expressing the *Campylobacter N*-glycan, SKI41 = backbone control, ∗ = significant

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